Assembly of JC Virus-Like Particles in COS7 Cells

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JC virus lacks an appropriate cell line to support virus replication. The establishment of a JC pseudovirus assembly system could play an alternative role for a virus culture system. COS7 cells and a transfer vector, pcDL-SRα296, were used to express JC viral structural genes. VP231-SRα, which encodes VP2/VP3 and VP1, but lacks 137 bp of the 5′-terminus of agnogene, showed both efficient nuclear migration and quantitative expression of the major capsid protein VP1. JC pseudovirus assembly was observed in the nucleus of VP231-SRα transfected cells. Evidence of JC pseudovirus assembly is presented. The further utilization of this system, which includes a study for the viral morphogenesis, serological diagnosis, as well as the potential application for gene transfer vector, is discussed. ***J. Med. Virol. 51:265–272, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: JC virus; pseudovirus assembly; VP231-SRα; COS-7

INTRODUCTION

JC polyomavirus (JCV) is responsible for the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML) [Major et al., 1992]. It is a member of the Polyomavirus genus, which includes SV40 and Polyomavirus. The virus is double-stranded DNA of approximately 5,130 bp. The viral genome encodes early proteins, large T and small t antigens, and late structural proteins, agnoprotein, VP1, VP2 and VP3 [Frisque et al., 1984]. Like other Polyomaviruses, the viral structural proteins are synthesized in the cytoplasm, and subsequently migrate to the nucleus to form a viral particle [Wychowski et al., 1986, 1987; Gharakhanian et al., 1987]. VP1 is considered a major capsid protein [Salunke et al., 1986; Garcea et al., 1987]. A major limitation for studying JC virus has been the lack of an adequate virus culture system. So far, primary cultures of human fetal glial cells have been the most efficient system to support viral replication [Padgett et al., 1971]. Several attempts have been made to propagate the virus in immortalized cell lines [Major et al., 1985; Mandl et al., 1987; Akatani et al., 1994]. However, even when the virus can be cultured, prolonged periods of growth are necessary to produce sufficient virus titers.

Recently, pseudovirus assembly for other viruses has been reported [Hwang et al., 1994; Gonzalez and Affranchino, 1995]. With JC virus, it is essential to demonstrate quantitative expression of VP1 with efficient nuclear migration to establish an in vivo pseudovirus assembly system. Such a system plays an alternative role for virus culture system, and should be available for studying viral morphogenesis.

The establishment of a JC pseudovirus assembly system is described using COS7 cells [Gluzman, 1981] and pcDL-SRα296 [Takebe et al., 1988]. This system could be used in the serological diagnosis for JC virus infection and as a gene transfer vector for therapeutic trials.

MATERIALS AND METHODS

Virus Genome and Expression System

JC virus Tokyo-1 strain was prepared as described previously [Nagashima et al., 1981; Matsuda et al., 1987]. The sequence was partially confirmed to be JC virus type II, with a difference of several bases from prototype MAD-1 [Frisque et al., 1984] as described previously [Ault and Stoner, 1992].

COS7 cells, an established line of SV40-transformed monkey kidney cells [Gluzman, 1981] obtained from ATCC (CRL 1651), were grown at 37°C as monolayers in Eagle's minimum essential medium (Nissui Pharmaceutical Co., LTD) supplemented with antibiotics and 10% fetal bovine serum. The vector pcDL-SRα296 used in this study has been previously described [Takebe et al., 1988].

Recombinant Plasmids Construction

The steps to construct the recombinant plasmids used in this study are shown schematically in Figure 1. For simplicity, the prefix pJCT has been omitted from the

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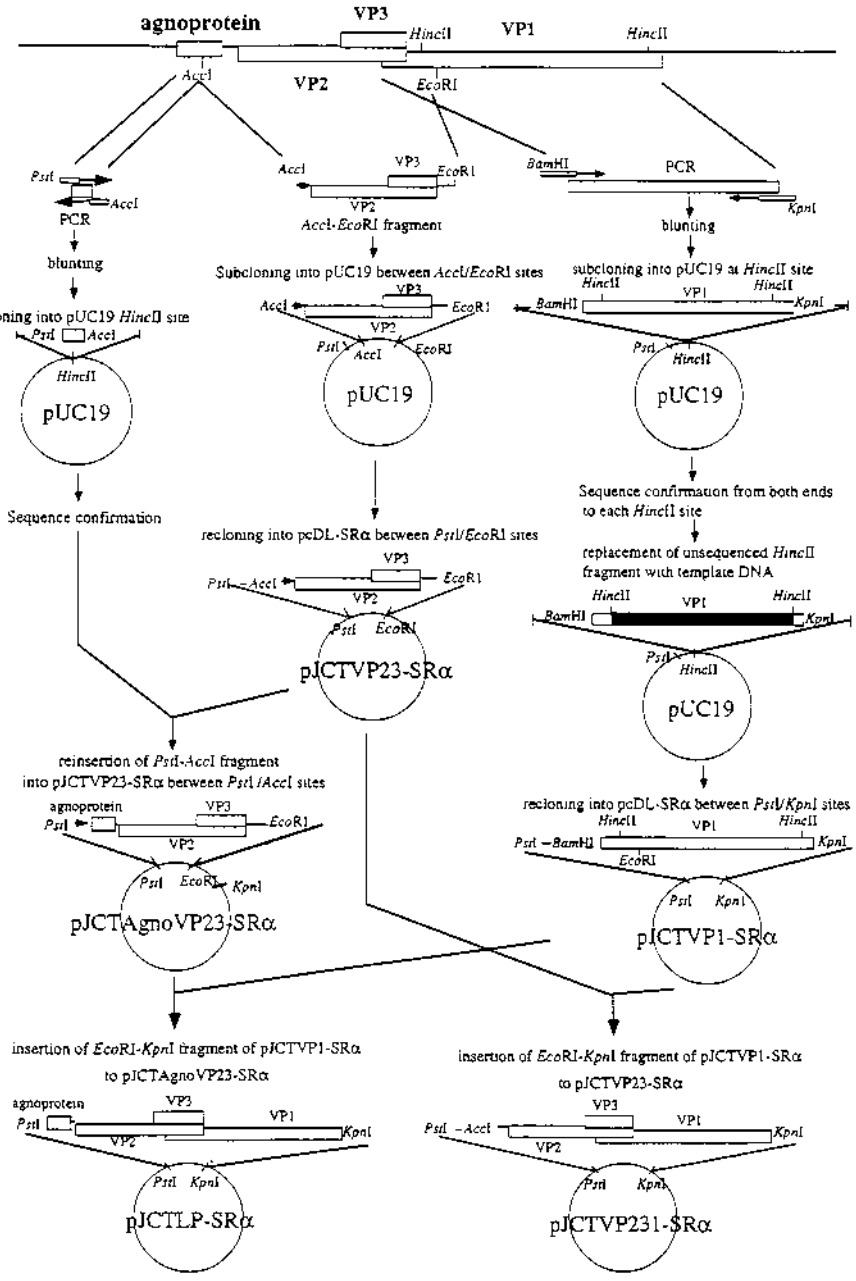


Fig. 1. Construction of JC virus late protein expression plasmids. The late proteins are agnoprotein, VP1, VP2 and VP3. A schematic diagram for construction of pJCTVP23-SRα, pJCTAgnoVP23-SRα, pJCTVP1-SRα, and pJCTVP231-SRα is shown. Details of the procedures are described in Materials and Methods.

names of the plasmid constructs. The individual procedures for each expression plasmid (Fig. 2) are given below.

VP1-SR*α *(VP1). The entire VP1 coding region was amplified by PCR using primers complementary to each end of VP1, and contained a *Bam*HI recognition site for the 5′-end (PRVP15′*Bam*HI; 5′-GAAGGATCCGAAGATGGCCC-3′) and a *Kpn*I recognition site for the 3′-end (PRVP13′*Kpn*I; 5′-GGGGTACCTTACAGCAT-3′), respectively. The PCR product was first blunted, and then subcloned into the pUC19 *Hinc*II site. The sequence of the subcloned PCR product was confirmed from both ends of VP1 to the *Hinc*II sites at map units 0.973 and 0.153, by using forward and reverse M13 universal primers. The unsequenced *Hinc*II fragment of VP1 between 0.973 and 0.153 was replaced with template DNA, so that the possibility of misreading the PCR product could be completely eliminated. The insert, which is the entire ORF of JC virus VP1, was recloned into pcDL-SRα296 between the *Pst*I and *Kpn*I sites,

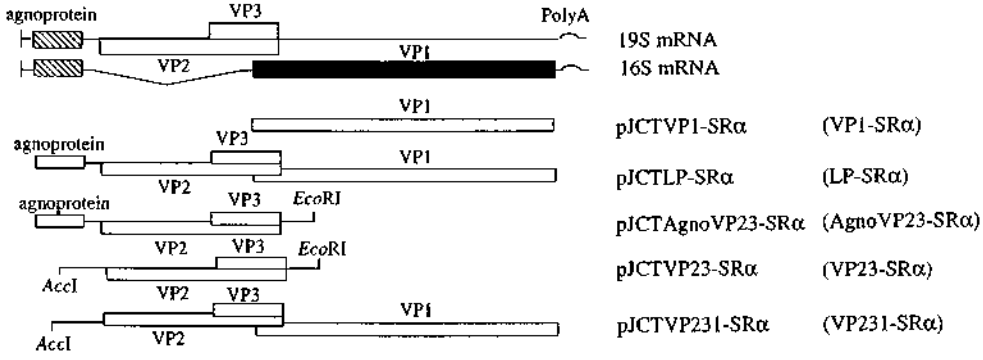


Fig. 2. Schematic representation of the expression vectors used. Various regions of late protein's open reading frames were inserted downstream of the SRα promoter. The details are described in Materials and Methods and a schematic explanation is given in Fig. 1.

using the *Pst*I site of pUC19's multiple cloning site (MCS) as a linker.

VP23-SR*α*(VP23). The *Acc*I-*Eco*RI fragment, which includes the 3′-terminus of the agnogene and the whole coding region for VP2/VP3, was first subcloned into pUC19 between the *Acc*I-*Eco*RI sites, then recloned into pcDL-SRα296 between the *Pst*I and *Eco*RI sites, by using pUC19's MCS as a linker. VP23-SRα lacks 137 bp of the 5′-terminus of the agnogene, including the ATG codon.

AgnoVP-SR*α*(AgnoVP23). The region from the agnogene ATG codon to the *Acc*I site (137 bp) was amplified by PCR, using primers designed to introduce a *Pst*I site at the 5′-terminus (PRAgno5′*Pst*I; 5′-CCCTGCAGATGGTTCTTCGCC-3′) and an *Acc*I site at the 3′-terminus (PRAgno3′*Acc*I; 5′-CCGTCTACACTGTCTTCACC-3′). The sequence was subsequently confirmed. The PCR product was reinserted into VP23-SRα downstream of the SRα promoter, between the *Pst*I and *Acc*I sites, resulting in the construct AgnoVP23-SRα, which encodes the agnogene and VP2/VP3.

LP-SR*α *(LP). This plasmid was designated "late proteins," as it encodes the sequences of agnoprotein, VP2/VP3 and VP1. Plasmid Agno VP23-SRα (see above) was first opened with digestion of *Eco*RI and *Kpn*I, followed by the insertion of the *Eco*RI-*Kpn*I fragment of VP1-SRα to yield LP-SRα.

VP231-SR*α *(VP231). The same procedure employed for the construction of LP-SRα was used, but VP23-SRα replaced AgnoVP23-SRα. VP231-SRα possesses a 5′-truncated agnogene, VP2/VP3 and VP1.

Transfection

COS7 cells were transfected utilizing Lipofectamine (GIBCO BRL) according to the manufacturer's instructions. Approximately 100 ng DNA with 1 μl Lipofectamine was used onto almost 10^4 cells for immunocytochemical assay, and 1 μg DNA with 7 μl Lipofectamine onto 10^5 cells for radioimmunoprecipitation.

Antibody and Indirect Immunofluorescence (IF)

For detecting transiently expressed VP1, an anti-JC virus antibody was used. JC virus, cultured in primary human fetal glia cells, was inoculated into rabbits, and the antiserum prepared 3 weeks after the first injection. The antibody was affinity purified and its specificity tested with PML brain tissues and JC virus Tokyo-1 infected primary human fetal glia cells [Nagashima et al., 1981]. The antibody was found to be highly specific for epitopes of VP1; it does not recognize epitopes of agnoprotein, VP2 and VP3 (data not shown).

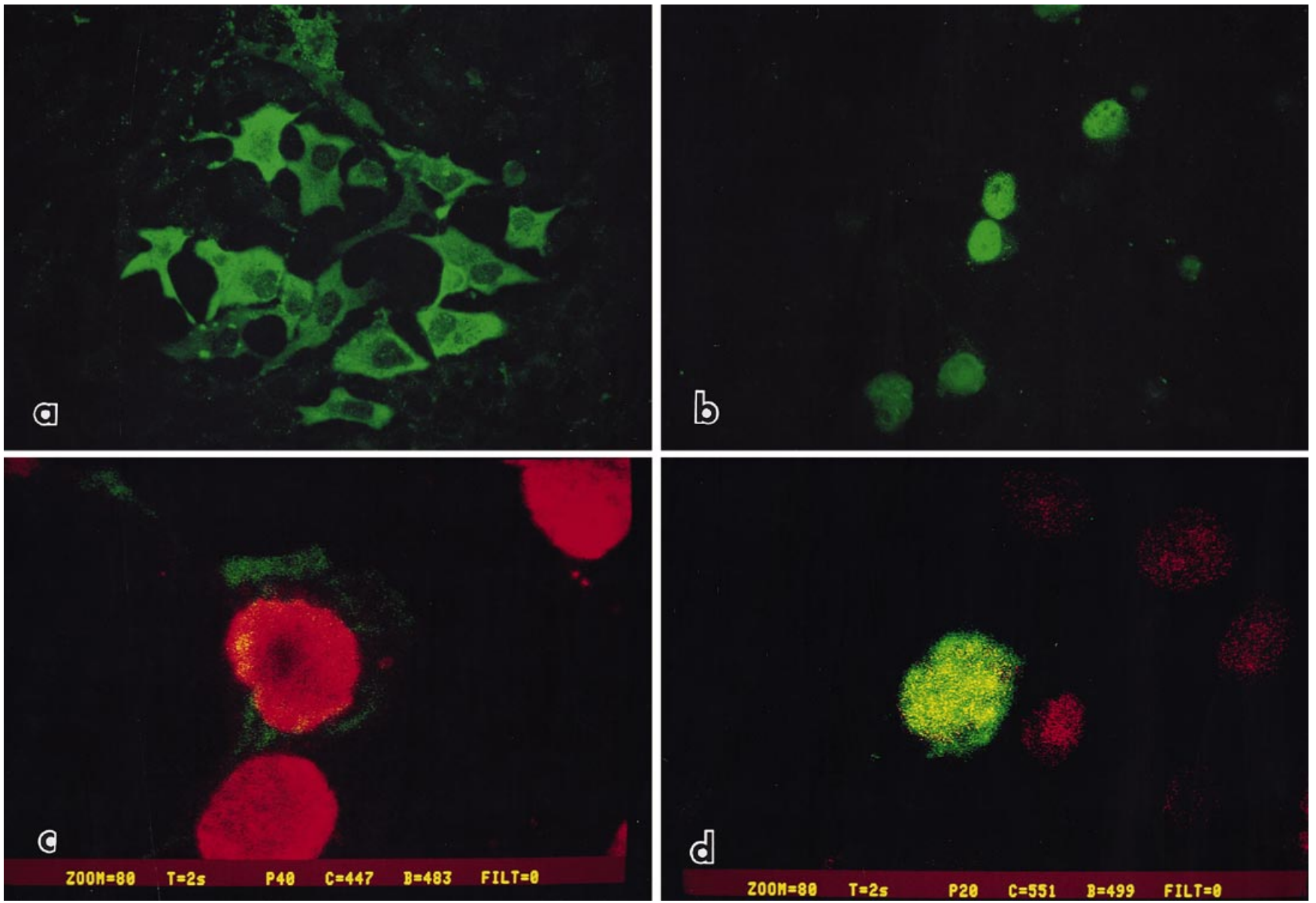
COS7 cells were harvested 72 h post-transfection. The cells were washed with phosphate-buffered saline (PBS), fixed for 5 min in cold acetone, and incubated for 1 h at 37°C with the anti-JC virus antibody (1:100 dilution). Subsequently the cells were washed again with PBS, and then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Cooper Biomedical). Following washing with PBS (5 min, three times), the cells were mounted with GelTol™ Aqueous Mounting Medium (Lipshaw/Immunon TM) and examined with the fluorescence microscope(Axioplan, ZEISS).

Confocal Microscopic Examination

The cells were immunostained doubly with the anti-JC virus antibody and an anti-nuclear antibody, using the same conditions as for IF. The anti-nuclear antibody was prepared from the purified sera of patients with systemic lupus erythematosus. FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-human IgG (CAPPEL) were the respective second antibodies. The doubly stained cells were examined with Confocal Laser Scan Microscope (LSM) (ZEISS, Germany).

Radioimmunoprecipitation (RIP)

The cells, 60 h post-transfection, were starved for 4 h in methionine- and cystine-free medium and then labeled for 20 h with 150 μCi/ml of ^{35}S Protein Labeling Mix (NEN). The labeled cells were solubilized in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate), then incubated with anti-JC virus antibody for 16–20 h at 4°C. The antigen-antibody complexes were captured with protein A-Sepharose beads (Sigma) and washed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with RIPA buffer. The ^{35}S-labeled antigen was released by addition of an equal volume of 2 × SDS-PAGE loading buffer (100 mM Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 4% SDS, 0.2% BPB, 20% glycerol) followed by incubation at 97°C for 5 min. The samples thus prepared were electrophoresed through 10% polyacrylamide gels (TEFCO Co.) using a constant current of 20 mA. After electrophoresis, the gels were fixed for 15 min with 40% methanol and 10% acetic acid and then prepared for fluorography (Amplify™, Amersham). The radiolabeled proteins were detected by exposure of the gels to Kodak X-OMATAR film at −80°C.

Electron Microscopy

The cells were harvested 72 h post-transfection, washed with PBS, fixed with 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.1% phosphate buffer (pH 7.3), and embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with an H-800 electron microscope (Hitachi).

RESULTS

Nuclear Migration of VP1

Expression and subcellular localization of VP1 were assessed by an immunofluorescent assay (IF). COS7 cells transfected with VP1-SRα and those transfected with LP-SRα were examined. In cells transfected with VP1-SRα, VP1 expression was mostly cytoplasmic, with occasional nuclear staining (Fig. 3a). Distinct nuclear staining was evident in about 10% of the VP1-positive cells. By contrast, when cells were transfected with LP-SRα, VP1 was detected only in the nuclei (Fig. 3b). These observations were corroborated by confocal microscopy and double immunostaining with the anti-JC virus and anti-nuclear antibody, using FITC and rhodamine as the respective chromogens (Figs. 3c,d). The results confirmed dominant cytoplasmic localization of VP1 in VP1-SRα transfected cells, and nuclear localization of VP1 in LP-SRα transfected cells. Agno VP23-SRα and VP23-SRα were co-transfected with VP1-SRα respectively. Transfection of VP231-SRα was also conducted. In each experiment, VP1 was found to accumulate in the nucleus (data not shown).

Quantitative Expression of VP1 by VP231-SRα

Qualitative and quantitative VP1 expression was assessed by a radioimmunoprecipitation (RIP). A 40 kDa molecule of VP1 was detected. The quantity of VP1 differed between VP1-SRα, LP-SRα and VP231-SRα transfected cells. With LP-SRα, less than 5% of the cells were VP1 positive by IF. However no VP1 was detected by RIP, because of the relatively low number of VP1-expressing cells. By comparison, with VP231-SRα approximately 70–80% of the cells were VP1 positive by IF, and RIP showed a strongly positive band of VP1. Cells transfected with VP1-SRα, as well as those co-transfected with VP1-SRα and VP23-SRα, were both less efficient in expressing VP1 compared with VP231-SRα. Thus, the highest VP1 expression was obtained by VP231-SRα (Fig. 4).

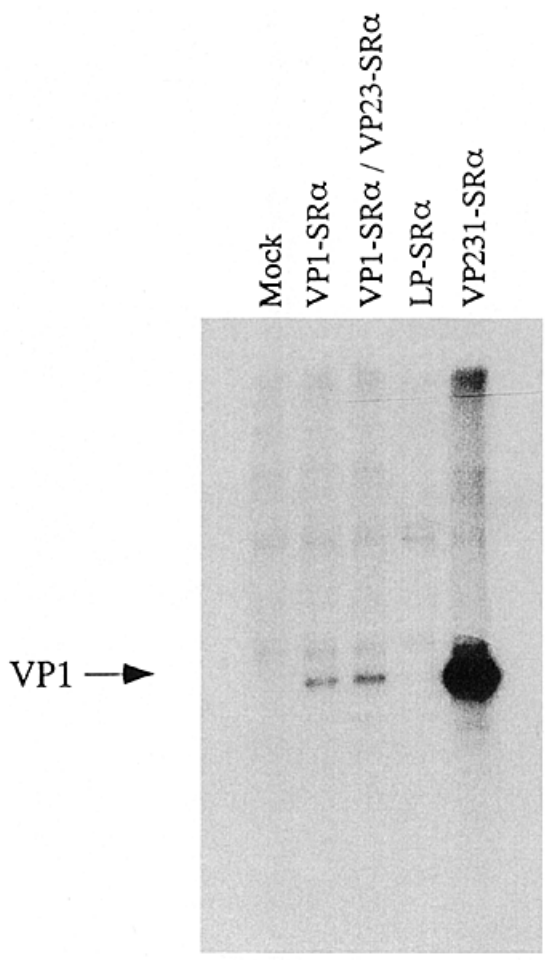

Fig. 4. Variation of VP1 expression levels among three expression plasmids, VP1-SRα, LP-SRα and VP231-SRα. Co-transfection was also performed with VP1-SRα and VP23-SRα (VP1-SRα/VP23-SRα). Note the striking increase in VP1 expression elicited by VP231-SRα.

Ultrastructural Analysis of JC Virus Particle Formation

This portion of the study was carried out with COS7 cells transfected by VP231-SRα. Filamentous profiles with a thickness of approximately 30 nm and round particles with a diameter of 40 nm were present in the nucleus of cells transfected with VP231-SRα (Fig. 5). The observed ultrastructural features were identical to those of JC virus (Tokyo-1 strain) propagated in primary human fetal glia cells [Nagashima et al., 1981]. Viral particles were not identified in the cytoplasm.

Fig. 3. Subcellular localization of JC virus VP1. COS7 cells were transfected with VP1-SRα **(a,c)** and LP-SRα **(b,d)**. In a and b, fixed cells were stained with rabbit anti-JC virus antibody and then with FITC-conjugated goat anti-rabbit IgG. In c and d, cells were doubly stained first with rabbit anti-JC virus and then with human anti-nuclear antibodies, followed by FITC-goat anti-rabbit IgG and rhodamine-conjugated goat anti-human IgG, respectively. Conventional immunofluorescence microscopy: a and b; confocal microscopy: c and d.

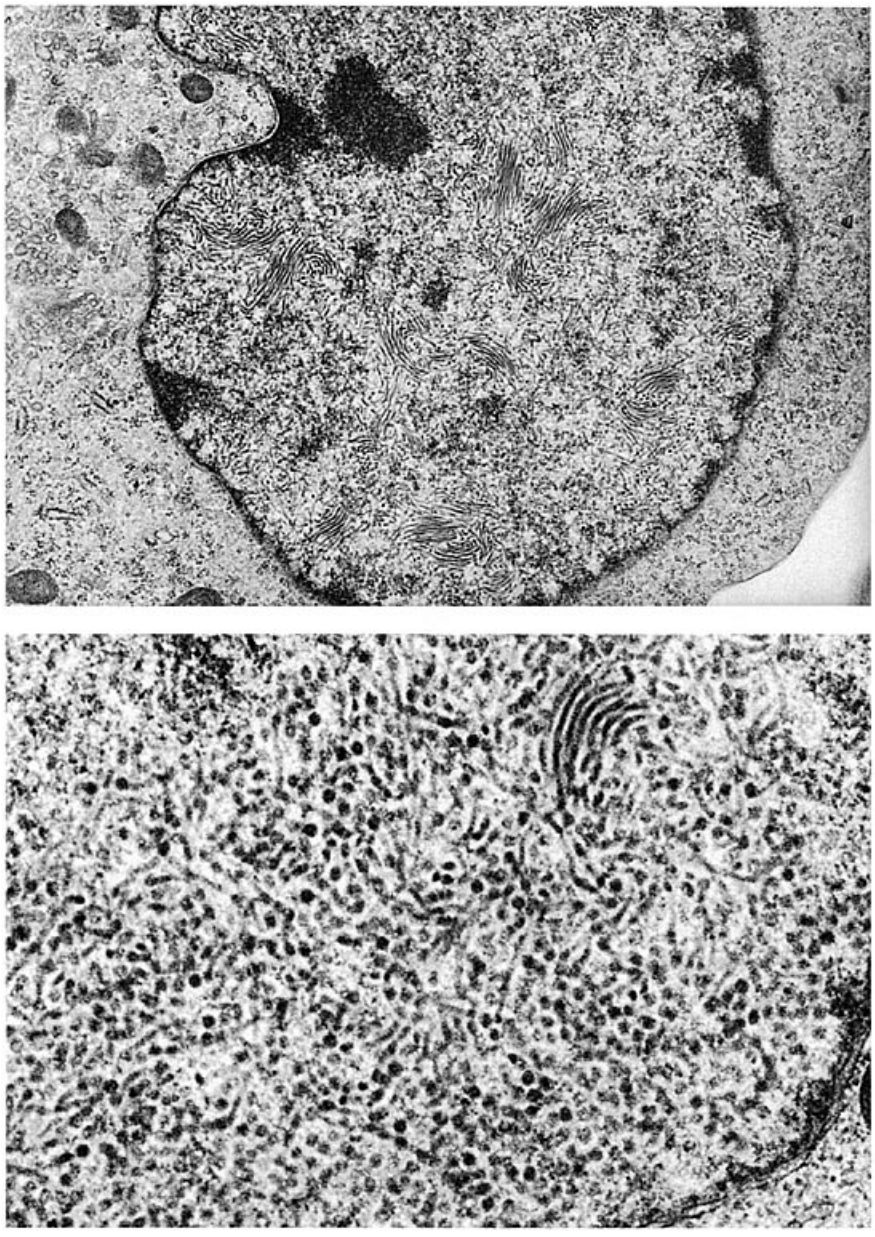

Fig. 5. Detection of JC virus particles. Both icosahedral particles and filamentous structures can be seen in the nucleus of a COS7 cell transfected with VP231-SRα. There are no particles in the cytoplasm. **Top**, ×15,000; **bottom**, ×60,000.

DISCUSSION

We provide ultrastructural evidence of JC pseudovirus formation in the nucleus of COS7 cells transfected with VP231-SRα. VP1, expressed by VP231-SRα, showed efficient migration to the nucleus and quantitative expression. In the Polyomavirus genus, structural proteins are synthesized in the cytoplasm, and migrate to the nucleus where they form viral particles. Nuclear migration of viral proteins of SV40 has been well studied [Kalderon et al., 1984a,b; Lanford and Butel, 1984]. There is a specific sequence, called nuclear localization signal (NLS), for each structural protein [Wychowski et al., 1986, 1987; Gharajhanian et al., 1987]. An NLS of SV40 VP1 has been mapped to its first eight N-terminal amino acids [Wychowski et al., 1986]. However, it would appear that this residue cannot function as an independent nuclear transport signal, because it does not promote entry of a non-nuclear protein into the nucleus. Recent studies have also shown that VP2/VP3 interacts with a nuclear targeting defective mutant of VP1 to facilitate its nuclear localization [Ishii et al., 1994]. Our results show that this situation also applies to JC virus VP1. JC virus VP1 has a sequence with high homology to the NLS of SV40 VP1 (Ala-Pro-Thr-Lys-Arg-Lys-Gly-Ser), differing by one amino acid at the end of the sequence (Ala-Pro-Thr-Lys-Arg-Lys-Gly-

Glu). But in our system, the nuclear migration of JC virus VP1 was poor in the absence of other structural proteins, specifically VP2/VP3 (Fig. 3). The sequence of the putative NLS of VP1, as well as evidence for an interaction between VP2/VP3 and VP1 for nuclear migration, has yet to be determined for JC virus.

VP1 expression varied between recombinant plasmids, which are VP1-SRα, LP-SRα and VP231-SRα (Fig. 4). VP231-SRα, as well as LP-SRα, was supposed to demonstrate lower VP1 expression than VP1-SRα, for two reasons as follows. One is that this plasmid has at least two extra ATG sequences upstream of VP1 ORF (one is for VP2, and the other for VP3). Protein expression is generally decreased when excessive ATG sequences are located upstream of the ORF. The other reason is that there is a relatively long distance from the SRα promoter to the ORF of VP1. However, our finding is different. LP-SRα demonstrated lower VP1 expression, but VP231-SRα showed a significant amount of VP1, even though Kozak's theory has to be considered seriously [Kozak, 1987]. We cannot explain such a result based on our data so far; however, it is possible there is some regulation mechanism for VP1 expression. Agnoprotein was first discovered in SV40 [Jay et al., 1981], when the agnogene was predicted to regulate the expression of late structural proteins. Premature termination signal with the putative attenuation loops, which is analogous to that of bacteria, was detected [Hay et al., 1982]. Such a signal is encoded in the ORF of the agnogene, 93–95 bp downstream of the major initiation site. JC virus itself has a similar motif. VP231-SRα is deleted 137bp of the 5′-terminus of agnogene, where such a motif is encoded. Then, VP231-SRα might hit remarkable increase of VP1 expression, compared with LP-SRα (Fig. 4).

JC viral morphogenesis is not completely known; however, VP231-SRα did form virus particles (Fig. 5). The filamentous and icosahedral particles seen in the nuclei of COS7 cells were very similar to those found in JC virus–infected primary human fetal glia cells and in oligodendroglial cells of patients with progressive multifocal leukoencephalopathy [Nagashima et al., 1981]. There are several possible applications of this JC pseudovirus assembly system. First, several hypotheses of viral morphogenesis, described above, can be confirmed using this system. Such a study might give us an answer of its inefficient replication in the virus culture systems. COS7 cells, a monkey kidney cells line, might be useful to assess viral replication during latency, because JC virus hidden in human kidney is excreted to urine of healthy carriers. Second, the pseudovirus must be useful for serological diagnosis of JC virus infection. Enzyme-linked immunosorbant assay (ELISA) and hemaggulutination inhibition assay (HAI) are common methods to diagnose seropositive patients. The pseudovirus can provide useful antigens for these assays. Third, a therapeutic application of this pseudovirus as a transfer vector might be possible. In this system pcDL-SRα296 and COS7 cells are utilized. The SRα, a chimeric product of SV40's early promoter and the long terminal repeat of HTLV-I, has an SV40 replication origin. So, COS7 cells, stably expressing SV40 T antigen, are advantageous to amplify pcDL-SRα296, because T antigen promotes the viral replication. The SRα, moreover, possesses an encapsidation signal of SV40, which packages DNA into virus particles [Colomar et al., 1993; Oppenheim et al., 1994]. The Polyomaviruses uncoat their DNA in the nucleus of host cells. Then SV40 capsid carrying foreign DNA, successfully transfected on a hemopoietic cell line [Oppenheim et al., 1986]. It is yet uncertain whether JC pseudovirus can be useful as a vehicle. However, JC virus vector might be useful to treat certain disorders in the central nervous system. The JC pseudovirus assembly system is worthy of study for future utilization.

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